Rac and Bcr regulate phagocytic phoxes

The NADPH oxidase of phagocytes is essential for defence against invading microorganisms. The small GTPase Rac seems to be critical for the activation and deactivation of the phox proteins that make up this enzyme.

Small GTPases related to the oncoprotein Ras regulate a diverse array of intracellular processes, from signal transduction to vesicle trafficking [1]. All act as molecular switches, interacting with different proteins according to whether the GTPase is bound to GTP or GDP. In some cases, the GTP-bound form is active and transmits a signal while the GDP-bound form is inactive. Ras, for example, interacts with its target proteins Raf and phosphoinositide (PI) 3-kinase only when Ras is bound to GTP [2]. In other cases, such as in the regulation of vesicle trafficking by Rab proteins, cycling between GTP- and GDP-bound forms is essential for the process to occur [3]. Recently, rapid progress has been made in unravelling the involvement of Rac, a member of the Ras superfamily, in regulating the NADPH oxidase of phagocytic cells.

The NADPH oxidase produces superoxide anions (O_2^-) in response to a number of inflammatory stimuli associated with microbial infection [4]; the superoxide anions are then released into phagosomes as part of the cell's weapons aimed at killing engulfed bacteria. The active membrane component of the NADPH oxidase is cytochrome b_{558}, which consists of two subunits, gp91^{phox} and p22^{phox}. In addition, three cytosolic proteins, p47^{phox}, p67^{phox} and Rac, are required for the activation of NADPH oxidase in cell-free assays (Fig. 1). The four phox proteins are expressed principally in phagocytic cells and in B lymphocytes, although mRNAs encoding p22^{phox}, p47^{phox} and p67^{phox} have also been detected in human fibroblasts [5]. In addition, there are two Rac proteins, Rac1 and Rac2, which are 95 % identical to each other at the amino-acid level, and both can activate the NADPH oxidase [6]. Rac1 is ubiquitously expressed, whereas Rac2 is expressed predominantly in cells of myeloid lineage, and is the predominant isoform in human neutrophils [7]. Rac has also been independently characterized as a regulator of membrane ruffling and lamellipodial extension in fibroblasts [8].

How does Rac regulate the activity of the NADPH oxidase? Although the precise mechanism is not known, it is clear that Rac must normally be bound to GTP to stimulate NADPH oxidase activity, and that the GDP-bound form of Rac is inactive [6]. In addition, Rac becomes associated with the plasma membrane when neutrophils are activated. GTP-bound Rac interacts specifically with an amino-terminal domain of p67^{phox}, suggesting that p67^{phox} is a target for regulation by Rac [9]. But Rac does not appear to interact with p67^{phox} prior to membrane association, as Rac translocation can occur independently of p47^{phox} and p67^{phox} — Rac

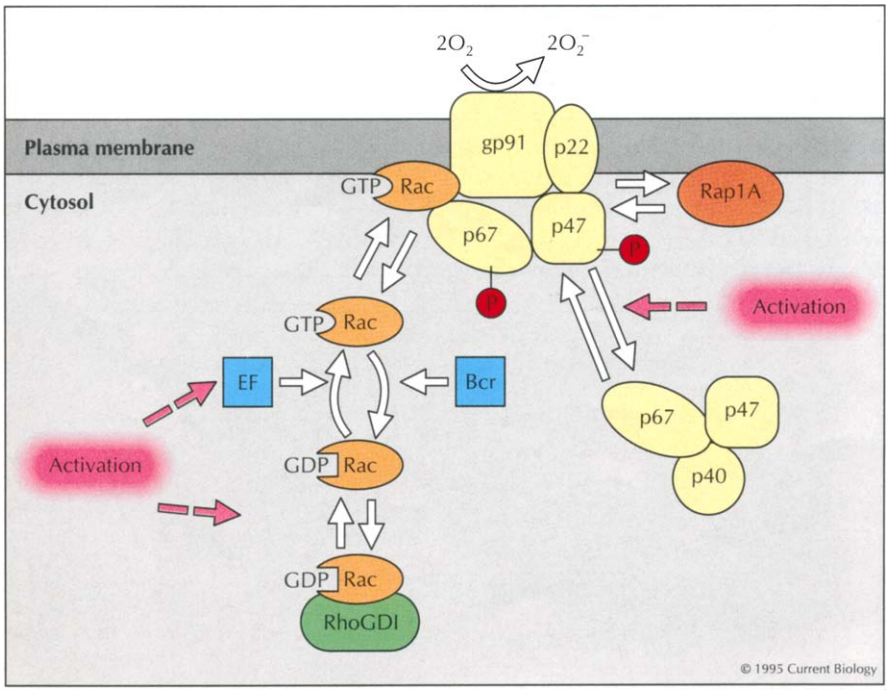

Fig. 1. Model for NADPH oxidase regulation in neutrophils. In resting neutrophils, Rac–GDP is complexed in the cytosol with RhoGDI, and p47^{phox} (p47) and p67^{phox} (p67) are complexed with p40^{phox} (p40). Upon activation, Rac–GDP dissociates from RhoGDI, an exchange factor (EF) stimulates exchange of GDP for GTP, and Rac–GTP associates with the plasma membrane. p47^{phox} and p67^{phox} become phosphorylated, dissociate from p40^{phox} and associate with the membrane-bound cytochrome b_{558} (gp91^{phox} and p22^{phox}), probably via interactions between p47^{phox} and p22^{phox}. Rac activates the membrane-bound complex, probably via interactions with p67^{phox} and gp91^{phox}, leading to production of superoxide anions (O_2^-). Rap1A also interacts with the cytochrome b_{558} and regulates its activity. NADPH oxidase activity can be down-regulated by hydrolysis of the GTP bound to Rac, and this reaction is stimulated by Bcr, acting as a GTPase activating protein (GAP).

translocates in cells lacking $p47^{phox}$ or $p67^{phox}$, and, conversely, the movement of Rac can be inhibited with tyrosine kinase inhibitors without affecting the translocation of $p67^{phox}$ and $p47^{phox}$ [7,10]. Optimal membrane association of Rac is, however, dependent on $gp91^{phox}$ expression, whereas translocation of $p67^{phox}$ requires $p47^{phox}$ and involves interactions between the Src homology 3 (SH3) domains of these two phox proteins and proline-rich motifs found in $p22^{phox}$ and $p67^{phox}$ [7,11]. These data suggest that Rac does not simply act by bringing together $p67^{phox}$ and $p47^{phox}$ with cytochrome b_{558} at the plasma membrane. Instead, it may act to assist positioning of these components at the membrane, or to stimulate enzymic activity through its interactions with $p67^{phox}$ and $gp91^{phox}$.

Rac is required for activation of the NADPH oxidase, but how is Rac itself activated in response to extracellular signals (Fig. 1)? In resting neutrophils, the majority of Rac is cytosolic, and it is complexed in its GDP-bound form with a protein known as RhoGDI, which binds to Rac and related proteins, including Rho and Cdc42Hs [6,12]. RhoGDI prevents both membrane association of Rac and nucleotide exchange on Rac, and thus an initial step in Rac activation is presumed to be its dissociation from RhoGDI. How this is achieved is not known, although there is evidence that some lipids can disrupt the Rac–RhoGDI complex [13], and one possibility is that the polyphosphoinositide products of the signalling enzyme PI 3-kinase are involved. In fibroblasts, it has been shown that PI 3-kinase acts upstream of Rac to mediate PDGF-induced lamellipodia formation [14]. In neutrophils, PI 3-kinase and the NADPH oxidase are simultaneously activated by several stimuli; furthermore, wortmannin, an inhibitor of PI 3-kinase, can prevent NADPH oxidase activation [15]. PI 3-kinase may therefore act upstream of Rac in this system as well. Rac activation also requires the exchange of GDP for GTP, a reaction catalysed by proteins known as nucleotide exchange factors. The protein(s) involved in regulating nucleotide exchange on Rac in response to extracellular stimuli have not been identified, although several proteins can stimulate nucleotide exchange on Rac-related proteins *in vitro*, including the oncoprotein Dbl [12].

The extent of the NADPH oxidase response must be precisely controlled in order to prevent excessive tissue damage. One way in which this can be achieved is by regulating the hydrolysis of GTP to GDP on Rac, a reaction catalysed by GTPase activating proteins (GAPs). A number of proteins with GAP activity for Rac have been identified, and these proteins all share a related GAP domain [12]. At least one of these GAPs, Bcr, has been shown to play an important role in down-regulating Rac activity in neutrophils [16]. Bcr was originally identified in the fusion oncoprotein Bcr–Abl, which is found as a result of chromosomal translocations in chronic myelogenous leukaemias. The Bcr GAP domain is active in promoting the GTPase activity of Rac and the related protein Cdc42Hs *in vitro*, and microinjection studies have shown that this domain can indeed down-regulate Rac activity in cells, as it inhibits growth factor-induced membrane ruffling [12].

In order to study the normal function of Bcr, mice lacking the *bcr* gene have recently been made [16]. These mice are viable, but their neutrophils show a dramatic increase in superoxide anion production in response to activating stimuli when compared to wild-type neutrophils. This defect leads to severe septic shock and tissue injury in the mice. There is also a three-fold increase in the amount of Rac2 that translocates to the plasma membrane upon neutrophil activation. These results strongly imply that Bcr normally down-regulates Rac, and that in its absence the proportion of active, membrane-bound Rac in stimulated cells increases and thus superoxide production is increased. It should be noted, however, that Bcr has other domains in addition to the GAP domain, including a Dbl domain — which is a potential nucleotide-exchange domain for Rac-related proteins — and a novel serine/threonine kinase domain. These domains may also play a role in down-regulating NADPH oxidase activity, and thus one function of Rac may be to bring Bcr to its active site at the oxidase complex. In this respect, it is interesting to note that no gross changes in actin organization were observed in either fibroblasts or neutrophils from the mice lacking Bcr, suggesting that the effects of Rac on the actin cytoskeleton do not involve Bcr. Instead, another GAP may be involved in regulating these functions of Rac.

Studies using cell-free assays indicate that only $p67^{phox}$, $p47^{phox}$ and Rac are required for activation of the membrane-bound NADPH oxidase, but the situation *in vivo* is likely to be more complex. For example, Rap1A, another Ras-related protein, is very abundant in neutrophil membranes and co-purifies with cytochrome b_{558}. Although Rap1A is not required for NADPH oxidase activation in cell-free assays, recent experiments indicate that it is indeed involved in NADPH oxidase function *in vivo*, as both activating and dominant-inhibitory mutants of Rap1A decrease the oxidative burst that occurs when neutrophils are activated [17]. This indicates that Rap1A is required to cycle between a GTP-bound and a GDP-bound form for the oxidase to be activated. It has been suggested that Rap1A acts to bring the cytochrome b_{558} in contact with another oxidase component, in a process of dynamic assembly and disassembly [17]. In addition, another cytosolic component, $p40^{phox}$, is found associated in a complex with $p47^{phox}$ and $p67^{phox}$ in resting neutrophils, and $p40^{phox}$ has been postulated to regulate interactions between $p67^{phox}$, $p47^{phox}$ and $p22^{phox}$ [18]. Finally, another potential target for Rac has been identified *in vitro* [19]. This potential target, $p65^{PAK}$, is a serine/threonine kinase that is activated by GTP-bound Rac; it is predominantly expressed in brain, but homologues may be expressed elsewhere. It is possible that Rac acts through $p65^{PAK}$ or a homologue to regulate NADPH oxidase activity via protein phosphorylation.

NADPH oxidase has provided a unique system for dissecting the function of a Ras-related GTP-binding protein, as a cell-free assay exists in which all the proteins have been characterized, and there is a clearly measurable biological response in cells. Although the mechanism whereby Rac activates the NADPH oxidase remains to be elucidated, substantial progress been made in characterizing the protein–protein interactions involved. This should be of general relevance to understanding how Ras-related proteins interact with their targets. In addition, phagocytes are an ideal model for studying whether the different functions of Rac in NADPH oxidase activation and actin reorganization are coordinately regulated

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Anne J. Ridley, Ludwig Institute for Cancer Research, University College School of Medicine, 91 Riding House Street, London W1P 8BT, UK.